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Genes Regulated by TorR, the Trimethylamine Oxide Response Regulator of *Shewanella oneidensis*

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The *torECAD* operon encoding the trimethylamine oxide (TMAO) respiratory system of *Shewanella oneidensis* is positively controlled by the TorS/TorR two-component system when TMAO is available. Activation of the *tor* operon occurs upon binding of the phosphorylated response regulator TorR to a single operator site containing the direct repeat nucleotide sequence TTCATAN$_4$TTCATA. Here we show that the replacement of any nucleotide of one TTCATA hexamer prevented TorR binding in vitro, meaning that TorR specifically interacts with this DNA target. Identical direct repeat sequences were found in the promoter regions of *torR* and of the new gene *torF* (*SO4694*), and they allowed TorR binding to both promoters. Real-time PCR experiments revealed that *torR* is negatively autoregulated, whereas *torF* is strongly induced by TorR in response to TMAO. Transcription start site location and footprinting analysis indicate that the operator site at *torR* overlaps the promoter −10 box, whereas the operator site at *torF* is centered at −74 bp from the start site, in agreement with the opposite role of TorR in the regulation of the two genes. Since *torF* and *torECAD* are positively coregulated by TorR, we propose that the TorF protein plays a role related to TMAO respiration.

Trimethylamine oxide (TMAO) is a small compound mainly found in aquatic environments (15). In a number of marine animals including fish and crustaceans, it stabilizes proteins against the denaturing effect of stresses such as hydrostatic pressure or high urea or salt concentration (20, 31, 32). This protective role is not yet clearly established for bacteria, but many of them can use TMAO as a terminal electron acceptor for anaerobic respiration (3, 28). For example, *Shewanella* strains, which are gram-negative bacteria with wide respiratory capacities, can reduce TMAO efficiently to generate energy during fish spoilage (13, 14, 16). The main TMAO respiratory pathway of *Shewanella* species comprises a periplasmic terminal reductase (TorA) containing a molybdenum cofactor and a pentaheme *c*-type cytochrome (TorC) anchored to the inner membrane (9, 12). The genes encoding the Tor pathway are clustered in the *torECAD* operon, and this operon is regulated by the TorS/TorR two-component system (6). When TMAO is available in the medium, the sensor TorS transphosphorylates the response regulator TorR which, in turn, activates the *torECAD* operon by binding to a single operator site in the operon promoter (12).

A similar Tor respiratory system is present in *Escherichia coli*, and its *torCAD* structural operon is also controlled by a TorS/TorR signal transduction system (18, 24). The *E. coli* TorS sensor detects the presence of not only TMAO but also immature TorC to allow optimal production of the structural components of the Tor respiratory system in inducing conditions (1, 19). The physiological relevance of this subtle negative autoregulation by apocytochrome TorC is probably that TorC maturation is the limiting step of the Tor system biogenesis (11). Overproduction of the *c*-type cytochrome maturation machinery relieves the negative autoregulation by increasing the extent of TorC maturation (1). In addition to the *torCAD* operon, TorR-P activates the *tnaLAB* operon encoding the tryptophanase (TnaA) and a low-affinity tryptophan permease (TnaB). The physiological reason for the coregulation of *torCAD* and *tnaLAB* is that the tryptophanase activity protects *E. coli* against the alkaline stress generated by the production of alkaline TMA during TMAO respiration (7). Indeed, TnaA reverses alkalinization by producing acidic products from L-tryptophan.

In this study, we show that TorR of *Shewanella oneidensis* activates *torECAD* and a new gene called *torF* (*SO4694*) and represses its own gene by binding to specific operator sites containing a direct repeat of the hexanucleotide sequence TT CATA separated by four nucleotides. *torF* encodes a protein that belongs to a new family of proteins of unknown function, and its coregulation with *torECAD* suggests that the TorF protein plays a key role in the TMAO respiratory system.

MATERIALS AND METHODS

Strains, media, and growth conditions. All strains of *S. oneidensis* used in this study are derivatives of strain MR1-R (6, 26). Strains SOR-3 and SOS-2 are, respectively, *torR* and *torS* insertion mutants. *S. oneidensis* was grown at 30°C in Luria-Bertani rich medium, complemented with 40 mM L-lactate and 20 mM HEPES as described by Myers and Myers (27). *E. coli* strains MC4100 and LCB436 [MC4100 but Δ(*torSTRCAD*)] were grown at 37°C in Luria-Bertani medium (12). To maintain plasmid selection in *E. coli*, ampicillin was added at a concentration of 50 μg/ml.

DNA manipulations. DNA preparation, restriction endonuclease digestion, purification, and ligation were carried out according to standard procedures. The transformation of *E. coli* was performed as described by Chung and Miller (8).

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TABLE 1. Synthetic oligonucleotides used in this study

Oligonucleotide	Sequence
WT	5′-TTTTAAAATTTTCATAATTTTTCATATATTGTCAAAGCC-3′
M1	5′-TTTTAAAATTTTGCTAATTTTTCATATATTGTCAAAGCC-3′
M2	5′-TTTTAAAATTTTCATAATTTTTGCTATATTGTCAAAGCCCATTCATCCC-3′
M3	5′-TTTTAAAATTGTCATAATTTTTCATATATTGTCAAAGCC-3′
M4	5′-TTTTAAAATTTGCATAATTTTTCATATATTGTCAAAGCC-3′
M5	5′-TTTTAAAATTTTGATAATTTTTCATATATTGTCAAAGCC-3′
M6	5′-TTTTAAAATTTTCGTAATTTTTCATATATTGTCAAAGCC-3′
M7	5′-TTTTAAAATTTTCAGAATTTTTCATATATTGTCAAAGCC-3′
M8	5′-TTTTAAAATTTTCATGATTTTTCATATATTGTCAAAGCC-3′
M9	5′-TTTTAAAATTTTCATAAGTTTTCATATATTGTCAAAGCC-3′
M10	5′-TTTTAAAATTTTCATAATTTTGCATATATTGTCAAAGCCCATTCATCCC-3′
Erev	5′-AAGAGTATGAAAATGATGAATCCCAG-3′
pR1	5′-CCAGCACACTATAGGCCATGTTC-3′
pR2	5′-CATAGTTTGTTAGCGTCCACC-3′
F1	5′-CGTGGAGAATCCGAAACCTTAG-3′
F2	5′-CCGCATACCAGCCTTGATTGTG-3′
F3	5′-CTACGCCTAGCTATCCATAAGC-3′
F5	5′-GAAAGTAACGCCCCAGTTAGC-3′
949A	5′-GATTAACGGATAGGTAAACGGG-3′
949B	5′-AGTGATTATGTGTCGATTAGCC-3′
C1	5′-CCTTAGGCGCTGTCAGCATTAG-3′
C2	5′-ATGCAGAAGGCTTCGGTATTGG-3′
16S1	5′-CGGACGGGTGAGTAATGCCTAG-3′
16S2	5′-GTAGGGCGTATGCGGTATTAGC-3′
R1	5′-AAGGTTATCGCGTGGTTGAGGC-3′
R2	5′-GCGTAACTCGCGAGTTAAGCTC-3′
R5	5′-CGCGCACGAATAACCACTTCGTCATC-3′
R7	5′-AAGCAAAATAAGAGAAACAGAACATG-3′
R8	5′-ATGAAAAAGTATGAATAATTCAGTT-3′
lacZ	5′-CGCCAGGGTTTTCCCAGTCACGAC-3′

Plasmid constructions. To create plasmid $pPTorR_{SO}$, we performed PCR by using *S. oneidensis* chromosomal DNA as a template and the primer pair pR1-pR2 (Table 1) to generate a DNA fragment extending from −182 to + 19 (nucleotide position relative to the translation start site of *torR*). The PCR product was cloned into the SmaI site of pGE593 (10), and the resulting plasmid ($pPTorR_{SO}$) was introduced into strain LCB436. The appropriate cloning orientation was determined by PCR. The absence of mutation in the cloned fragment was checked by DNA sequencing.

RNA preparation. RNA was prepared by using a High Pure RNA isolation kit from Roche Diagnostics according to the manufacturer's instructions but with the slight modification that the DNase I digestion step was carried out twice in order to diminish the quantity of contaminating DNA. When the RNA was prepared in order to perform real-time PCR experiments, an additional third step of DNase I treatment was carried out in solution with RNase-free DNase I (Amersham) between the two passages through columns.

Primer extension analysis. The transcription start sites of the *torR* and *torF* genes were determined in *E. coli* strain LCB436 carrying plasmid $pPTorR_{SO}$ and in *S. oneidensis* strain MR1-R, respectively. The strains were grown anaerobically in the presence of 50 mM TMAO until the culture reached an A_{600} of 0.5. Total RNA was then extracted. The oligonucleotides used as probes were end labeled with [γ-^{33}P]ATP (2,500 Ci/mmol) by using T4 polynucleotide kinase (Gibco-BRL) and purified with a QIAGEN QIAquick nucleotide removal kit. The primer extension reactions were performed with reverse transcriptase (Superscript II; Gibco-BRL). The sequencing ladders were generated with the same oligonucleotides used for the primer extensions.

RT PCR analysis. Reverse transcriptase PCR (RT PCR) was performed with the Promega Access system. The oligonucleotides used are indicated in Table 1 (see also Fig. 3). One microgram of purified RNA was denatured at 94°C for 2 min in the presence of the primers. Immediately afterwards, reverse transcription and 35 cycles of PCR amplification were carried out according to the supplier's protocol.

Real-time PCR. The relative abundance of the *torC*, *torR*, and *torF* transcripts of various *S. oneidensis* strains (MR1-R, SOR-3, and SOS-2) grown with or without TMAO (50 mM) was determined by real-time PCR. 16S rRNA was used as a reference standard. Real-time PCR was performed by using a LightCycler instrument and the LightCycler-FastStart DNA Master SYBR Green I kit (Roche Diagnostics) according to the manufacturer's instructions. Total RNA, extracted from *S. oneidensis* strains grown with or without TMAO (50 mM), was reverse transcribed by using random hexamers. cDNA (2 ng) was then mixed with 4 mM $MgCl_2$, a 0.1 μM concentration of each primer, and 2 μl of master mix in a 20-μl final volume. The primer pairs used to quantify the *torC*, *torR*, *torF*, and 16S rRNA gene expression levels were C1-C2, R1-R2, F1-F2, and 16S1-16S2, respectively (Table 1). PCR assay was carried out with one cycle at 95°C for 8 min, followed by up to 45 cycles at 95°C for 15 sec, 60°C for 10 sec, and 72°C for 10 sec. The fluorescence derived from the incorporation of SYBR Green I into the double-stranded PCR products was measured at the end of each cycle to determine the amplification kinetics of each product. The fit points method described by the manufacturer was then applied to the results. Briefly, a horizontal noise band was determined as well as a log line fitting the exponential portion of the amplification curve. The intersections of these log lines with the horizontal noise line identified the crossing points. These crossing points were determined for each gene in both growth conditions. The induction factor was calculated as follows: $2^{(\text{crossing point in absence of TMAO} - \text{crossing point in presence of TMAO})}$. The values were normalized by using values obtained with 16S rRNA. The real-time PCR experiments were performed three times with RNA samples prepared from independent cultures.

Preparation of the TorR protein of *S. oneidensis*. Overproduction of the TorR protein of *S. oneidensis* was achieved by growing 100 ml of strain MC4100 carrying plasmid $pR_{so}1$ (pBAD24 carrying the *torR* gene under the control of the arabinose-inducible promoter) (12). When the culture reached an A_{600} of 1, overproduction of the TorR protein was induced for 1 h with 0.2% arabinose. The cells were then harvested by centrifugation, and the pellet was resuspended in 5 ml of 40 mM Tris-HCl, pH 7.6. The cells were passed through a French press, and the extract was centrifuged at 14,000 rpm in a Sorvall RC5B centrifuge for 10 min. The supernatant was directly loaded on a heparin-Sepharose column (Amersham Pharmacia Biotech). The proteins were eluted with a step gradient of KCl from 100 mM to 1 M. TorR was purified near to homogeneity in the 400 mM KCl fraction.

Gel retardation assays. The DNA fragments were generated by PCR with the appropriate labeled and unlabeled primers. Labeling was carried out by using [γ-^{32}P]ATP (4,000 Ci/mmol) and T4 polynucleotide kinase (Gibco-BRL), and the labeled fragments were then separated from unincorporated nucleotides (QIAquick nucleotide removal kit; QIAGEN). Binding of TorR to labeled DNA fragments was carried out in a 4-μl reaction mixture containing 50 mM Tris-HCl

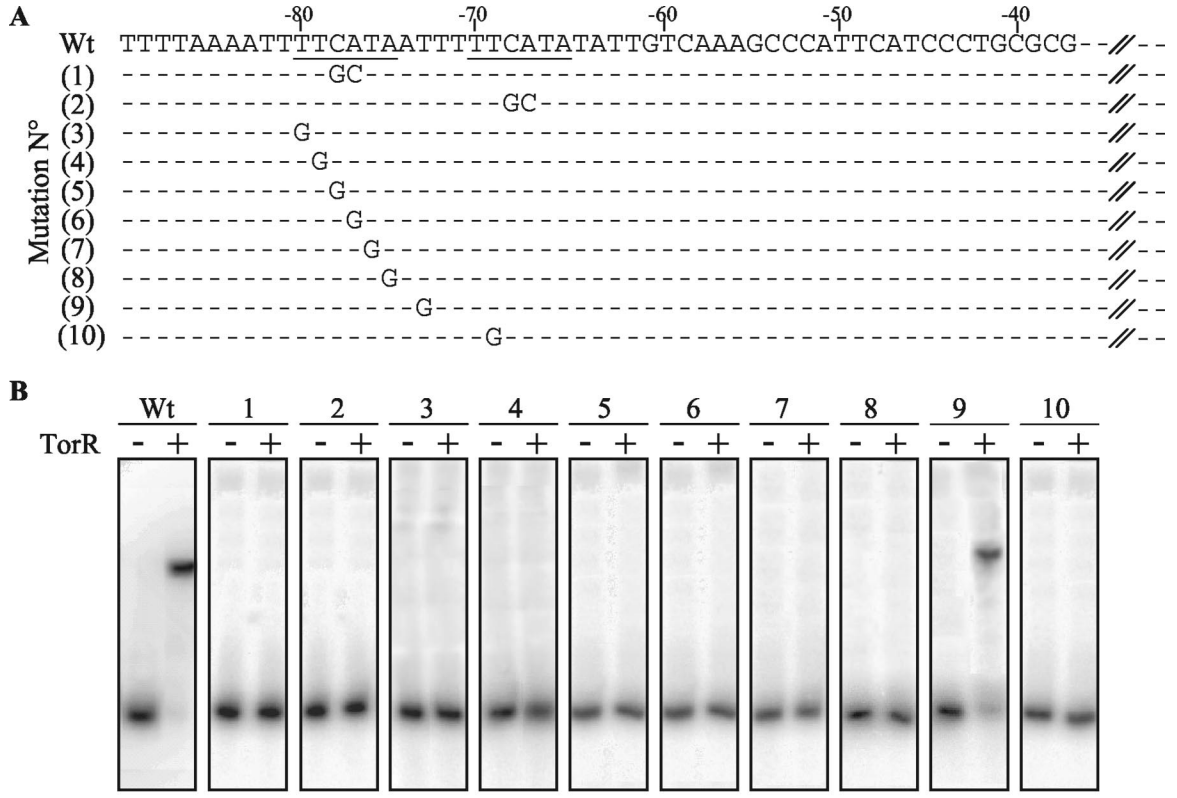


FIG. 1. Effect of mutations in the *torE* promoter region on the in vitro binding of TorR. (A) Representation of the wild-type and mutated promoter regions. The DNA fragments (209 bp) were obtained by PCR with Erev as the 3′ primer and WT, M1, M2, M3, M4, M5, M6, M7, M8, M9, or M10 as 5′ primers leading to the wild type and the corresponding mutated (1 to 10) promoter regions. Positions relative to the transcription start site are indicated above the sequences. The direct repeats are underlined. Only bases differing from the wild-type sequence are shown for the mutated fragments. (B) Gel shift analysis. The labeled fragments corresponding to the wild type and mutated (1 to 10) promoter regions were incubated in the absence (−) or presence (+) of a 1 μM concentration of purified TorR protein. Wt, wild type.

(pH 8), 1.25 mM EDTA, 0.25 M sucrose, 0.025% bromophenol blue, and 0.25 μg of poly(dI-dC) per μl. After 30 min at room temperature, the samples were loaded and run on a 12.5% polyacrylamide gel (Pharmacia Phast System). The gel was exposed for 3 h at room temperature on a phosphorimager screen.

DNase I footprinting. The same labeled DNA fragments as those used for the gel retardation assays, encompassing the *torR* (201 bp) or the *torF* (386 bp) regulatory regions, were generated by PCR from plasmid $pPTorR_{SO}$ and from MR1-R chromosomal DNA, respectively, with the appropriate labeled and unlabeled primers. The footprinting experiments were performed as follows. About 1 nM of probe was used in 50 μl of binding mix [10 mM Tris HCl (pH 7.5), 50 mM NaCl, 2.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 4% glycerol, and 30 ng of poly(dI-dC) per μl]. Different amounts of the purified TorR protein were then added. After 30 min of incubation at room temperature, DNase I was added (1 U; Promega), and the reaction was conducted for 1 min and then stopped by the addition of 140 μl of DNase stop solution (192 mM sodium acetate, 32 mM EDTA, 0.14% sodium dodecyl sulfate, and 64 μg of yeast RNA per ml). After phenol-chloroform extraction and DNA-ethanol precipitation, the pellets were resuspended in loading solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) and loaded on an 8% polyacrylamide–8 M urea electrophoresis gel. The location of the protected nucleotides was deduced by running a ladder with the products of the G+A cleavage reaction.

RESULTS AND DISCUSSION

TorR binds to a direct repeat of the hexanucleotide sequence TTCATA in the *torECAD* promoter. We have previously shown that TorR, the TMAO response regulator of *S. oneidensis*, induces the *torECAD* operon by binding to a single operator site located between positions −84 and −60 relative to the transcription start site (12). Inspection of this region revealed the presence of a direct repeat of the hexameric sequence TTCATA (Fig. 1). This tandem direct repeat could be the target of TorR because members of the OmpR family usually interact with direct repeats (5, 21, 22, 25). To test this hypothesis, we first changed the center of each hexamer (italicized) independently by a double mutation (TT*CA*TA→TT*GC*TA) and carried out a DNA-binding gel shift assay with labeled DNA fragments corresponding to the *tor* operon region from position −90 to +119 and purified TorR. As shown in Fig. 1, the DNA fragments containing the double mutation (mutations 1 and 2) were not retarded by a high concentration (1 μM) of TorR, whereas the wild-type fragment was. This preliminary result is consistent with the idea that each hexamer plays a key role in TorR binding. To study further the involvement of the TTCATA hexameric sequence in TorR binding, we replaced each nucleotide of the first hexamer with a guanine residue (Fig. 1). Strikingly, no retardation was observed for any of the six mutated fragments (mutations 3 to 8), meaning that each nucleotide of the first hexamer is essential for TorR binding. To confirm that the two hexamers play a similar role in TorR binding, we replaced one nucleotide of the second hexamer with a guanine residue. As expected, the mutated DNA fragment (mutation 10) was no longer retarded by TorR. In contrast, a point mutation T→G in the four-nucleotide region spacing the tandem repeats did not significantly affect TorR binding

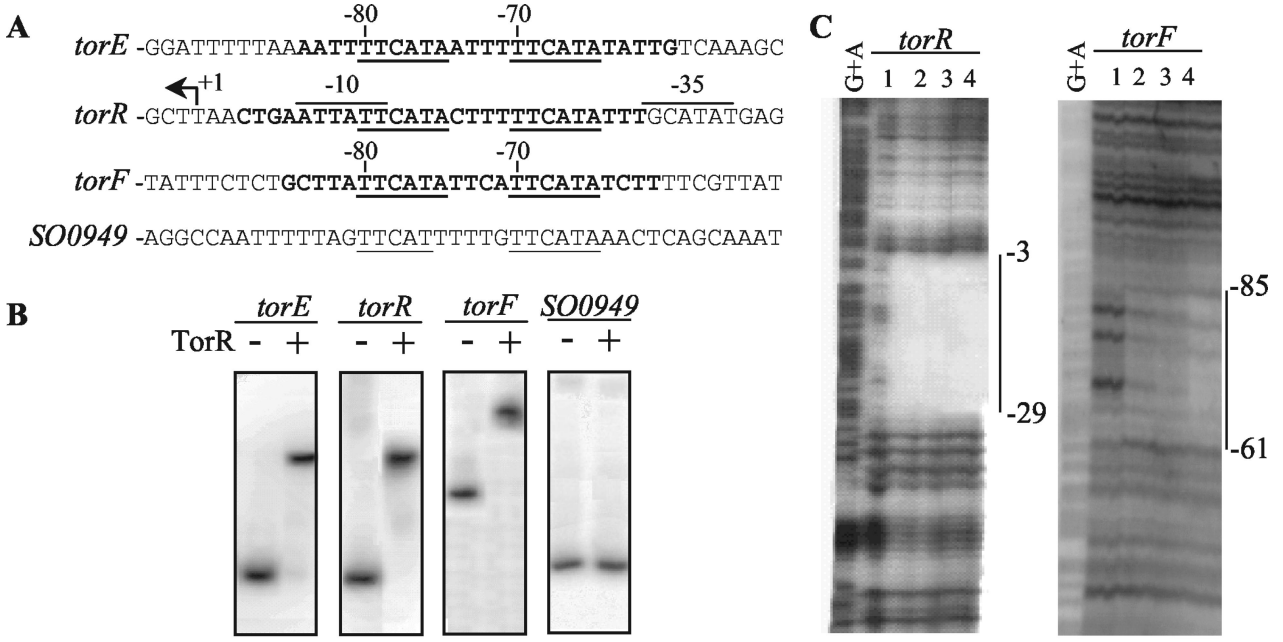


FIG. 2. (A) Alignment of the *torE*, *torR*, *torF*, and *SO0949* promoter regions. The regions protected by TorR are indicated in bold. The direct repeats are underlined. Positions relative to the transcription start sites are indicated above the sequences. For convenience, the complementary sequence of the *torR* promoter is presented. The direct repeat sequence of *SO0949* is centered at −165 bp from the initiation codon. (B) Electrophoretic gel shift analysis of TorR interaction with the *torE*, *torR*, *torF*, and *SO0949* promoters. The DNA fragments containing the *torE* (position −90 to + 119 relative to the transcription start site), *torR* (position −159 to + 42 relative to the transcription start site), *torF* (position −306 to + 80 relative to the transcription start site), and *SO0949* (position −234 to −54 relative to the initiation codon) promoter regions were obtained by PCR with the primer pairs Wt-Erev, pR2-pR1, F3-F5, and 949A-949B, respectively. The labeled fragments were used in gel shift experiments in the presence (+) or absence (−) of a 1 μM concentration of purified TorR protein. (C) Analysis of TorR binding to the *torR* and the *torF* promoter regions by DNase I footprinting experiments. The DNA fragments corresponding to the *torR* and the *torF* promoter regions were obtained by PCR by using the primer pairs labeled pR2-unlabeled pR1 and labeled F5-unlabeled F3, respectively. The labeled DNA fragments were digested with DNase I in the presence of the following concentrations of TorR protein: lane 1, no protein; lane 2, 0.25 μM; lane 3, 1 μM; and lane 4, 2.5 μM. The G+A sequencing ladders are shown, and the vertical bars indicate the protected regions.

(Fig. 1, mutation 9). The same results were obtained when TorR was preincubated with acetyl phosphate (data not shown). Together, these results strongly suggest that the DNA recognition site of TorR comprises at least the sequence TTCATAN$_4$TTC ATA, and we propose that like other members of the OmpR family, phosphorylated TorR binds as a dimer to its operator site, with each monomer interacting with one direct repeat (5, 29).

Binding of TorR to new promoters. The fact that we knew the specific nucleotide sequence recognized by TorR in the *torECAD* promoter prompted us to look for homologous nucleotide sequences within the genome of *S. oneidensis* in order to find new potential targets of TorR. By using the bioMotif utility (http://genetics.mgh.harvard.edu/doc/bioMotif/), we retrieved sequences homologous to the consensus sequence TT CATAN$_4$TTCATA, located in noncoding regions or in the beginning of coding regions. This survey revealed two additional sequences identical to the consensus and located upstream of the coding sequences of *torR* and of *SO4694* (hereafter called *torF*) and one sequence upstream of *SO0949* containing a single change in one hexamer (Fig. 2A). Since these sequences could be TorR binding sites, we checked whether the TorR protein was able to bind to them in vitro. Using a band shift assay, we observed DNA retardation for the promoter DNA of *torR* and *torF* but not for that of *SO0949* (Fig. 2B). This result shows that TorR binds to the *torR* and *torF* promoters, and it confirms that only one base change in one of the TTCATA repeat sequences prevents TorR binding. The same pattern of retardation was observed when TorR was preincubated with 100 mM acetyl phosphate, but the TorR affinity for the *torE*, *torR*, and *torF* promoter DNA was increased two- to threefold, indicating that phosphorylation of TorR increased its affinity for the DNA targets containing the consensus motif (data not shown).

The same pattern search approach was performed by using a five-nucleotide spacer between the two hexamers (consensus sequence, TTCATAN$_5$TTCATA). Indeed, an additional nucleotide in the spacer modifies the distance between the nucleotide motif of the hexamers from 10 to 11 bp, meaning that the same motifs are still present on the same side of the DNA helix and, thus, might still allow TorR binding. However, no sequence entirely matching the consensus was found within the *S. oneidensis* genome, and, out of the seven sequences containing a single base change in one hexamer, none allowed TorR binding in vitro (data not shown). These results support the idea that TorR recognizes highly specific sequences present at only a restricted number of sites on the chromosome of *S. oneidensis*.

To check that TorR binds to the consensus sequence TTC ATAN$_4$TTCATA in the *torR* and *torF* promoters, we carried out a DNase I footprinting analysis with the DNA fragments

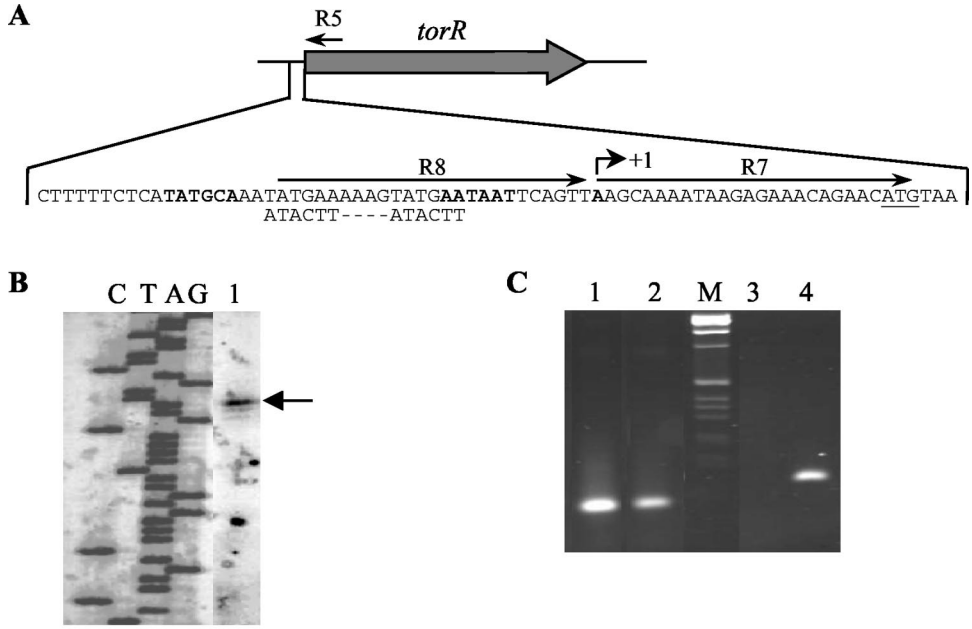


FIG. 3. (A) Nucleotide sequence of the *torR* promoter region. The transcription start site (+1) and the positions of oligonucleotides R5, R7, and R8 are indicated. The −10 and −35 regions are indicated in bold, and the ATG initiation codon is underlined. The direct repeat is indicated as a double-strand sequence. (B) Location of the transcription start point of gene *torR*. The labeled lacZ primer, complementary to the *lacZ* internal sequence, was annealed to total RNA from *E. coli* LCB436 carrying plasmid pPTorR$_{SO}$ and extended with RT (lane 1). The sequencing reactions were performed with the same primer as in the primer extension reaction. The samples were loaded on an 8% polyacrylamide–8 M urea electrophoresis gel. The arrow points out the transcription start site. (C) Analysis of the *torR* gene transcription by RT PCR followed by 2% agarose gel electrophoresis. The RT PCR was carried out with primer R5 and either primer R7 (lane 1 and 2) or R8 (lane 3 and 4). Lanes 2 and 3, RT PCR with 1 μg of total RNA from *S. oneidensis* MR1-R; lanes 1 and 4, control PCR with genomic DNA; lane M, 1-kb ladder from Gibco BRL.

used for the retardation experiments. As shown in Fig. 2C, in both cases a single region was protected against DNase I digestion when TorR was present. The protected regions extend over 25 to 27 nucleotides, and they cover the entire direct repeat sequences of the *torR* and *torF* promoters. This finding confirms that TorR recognizes operator sites containing a TT CATA repeat and suggests that it controls *torR* and *torF* gene expression.

Negative autoregulation of the *torR* gene. We tried to define the transcription start site of *torR* by primer extension experiments with RNA prepared from *S. oneidensis* MR1-R cells grown anaerobically with or without TMAO. These experiments were unsuccessful, probably because the amount of *torR* messenger was too low. To solve this problem, we fused the putative promoter region of *torR* to the promoterless *lacZ* gene of plasmid pGE593, and we introduced the resulting multicopy plasmid (pPTorR$_{so}$) into an *E. coli* strain (LCB436) from which the entire *tor* locus was deleted to avoid any interference. We then carried out primer extension by using RNA prepared from the plasmid-containing *E. coli* cells and a primer hybridizing to the 5′ end of *lacZ* (Fig. 3B). A transcription start site was located 23 bases upstream of the *torR* start codon. To confirm that the transcription start site of *torR* was identical in *E. coli* and *S. oneidensis*, we performed RT PCR by using RNA extracted from strain MR1-R and appropriate convergent oligonucleotide pairs (Fig. 3). When the upstream primer (R7) that hybridizes to the 5′ end of the potential *torR* messenger was used, a PCR product of the expected size was observed, but when an upstream primer (R8) complementary to the sequence just upstream of the putative transcription start site was used, no DNA fragment was amplified. The RT PCR experiment thus shows that the position of the *torR* transcription start site in *S. oneidensis* is identical or close to that defined by primer extension in *E. coli*.

A −10 promoter box (AATAAT) close to the *E. coli* −10 consensus sequence is correctly positioned relative to the start site, but the putative −35 box (TATGCA) is far from the *E. coli* −35 consensus box (TTGACA), supporting the idea that the *torR* promoter is weakly expressed in *S. oneidensis*. Moreover, one hexamer of the TorR operator site overlaps the −10 box, and, as a result, the TorR binding region which extends from position −3 to position −29 covers the −10 box (Fig. 2). Interaction of TorR with the *torR* promoter might thus hamper the correct binding of the RNA polymerase to this promoter, and, consequently, TorR might repress expression of its own gene. To test a possible negative autoregulation of the *torR* gene, we performed real-time PCR from total RNA extracted from MR1-R cells grown anaerobically with or without TMAO. The cDNA samples were synthesized by using random hexamers as primers, and the real-time PCR was carried out by using a *torR* specific primer pair (Table 1, R1-R2). Real-time PCR was also performed with a 16S-specific primer pair (16S1-16S2) to quantify the amount of 16S RNA in each sample, and the relative level of *torR* transcript was then normalized to that of the 16S RNA. As shown in Table 2, the amount of *torR* transcript decreased almost threefold when the cells were grown in the presence of TMAO, meaning that the expression of *torR* is negatively autoregulated, as expected from the in

TABLE 2. Analysis of the expression levels of *torC, torF*, and *torR* genes by real-time PCR

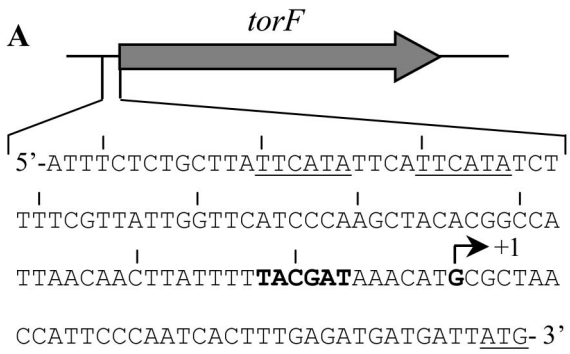
Gene	Induction factor[a] (with TMAO/without TMAO)	
	MR1-R	SOR-3
torC	21.6 ± 1.9	1.2 ± 0.3
torF	63.1 ± 2.8	1.6 ± 0.7
torR	0.35 ± 0.04	ND

[a] Values, normalized to the value of the 16S rRNA, were calculated as indicated in Materials and Methods. Values represent the means ± standard deviations of three independent experiments. ND, not determined.

vitro experiments (Fig. 2). Unfortunately, the control experiment with RNA extracted from the *torR* strain (SOR-3) was not feasible because the mutation in this strain corresponds to an ISSo2 insertion into the *torR* promoter region, leading to the absence of *torR* transcription (6). However, the *torR* transcript levels were similar in the *torS* strain (SOS-2) grown with or without TMAO (induction increased by a factor of 1.3 ± 0.3 [mean ± standard deviation]), thus confirming that the *torR* gene is negatively regulated by phosphorylated TorR.

In *E. coli*, the *torR* gene is also negatively autoregulated, but this autoregulation occurs even in a *torS* strain or in the absence of TMAO (2). In fact, the *E. coli torR* gene is always repressed because phosphorylated as well as unphosphorylated TorR binds to a high-affinity binding site overlapping the *torR* transcription start site (30). The situation is quite different in *S. oneidensis* since *torR* negative autoregulation occurs in the presence of TMAO and, thus, probably involves only the phosphorylated form of TorR. Consequently, TorR negative autoregulation maintains the TorR concentration at a low level whatever the growth conditions in *E. coli*, whereas in *S. oneidensis*, it decreases TorR production when TMAO is present in the medium. The reason for this subtle difference is unknown, but, in general, negative autoregulation has homeostatic properties and allows the production of a precise amount of regulator in the cell. Although more than one-third of the transcriptional factors are negatively autoregulated in *E. coli*, several response regulators proved to be positively autoregulated (17, 23). One proposal to explain positive autoregulation is that an increased concentration of a given response regulator is required in inducing conditions when the regulator controls many genes and must, therefore, bind to many targets at the same time (4). The restricted number of targets for TorR of *E. coli* and *S. oneidensis* might explain why TorR is negatively rather than positively autoregulated in both strains.

Activation of the gene *torF* (*SO4694*) by TorR. The transcription start site of *torF* was defined by a primer extension experiment with RNA prepared from MR1-R cells grown anaerobically with TMAO (Fig. 4). A single start site was located 34 bases upstream of the *torF* initiation codon, and a −10 promoter box (TACGAT) was found correctly positioned relative to the start site. In contrast, no putative −35 box could be found 16 to 18 bp upstream of the −10 box, but the TorR binding site is centered 74 bp upstream of the start site at a position compatible with that of an activator binding site (Fig. 2 and 4). To follow expression of *torF*, we carried out real-time PCR experiments from total RNA prepared from strains MR1-R and SOR-3 grown with or without TMAO. As shown in Table 2, *torF* expression was strongly induced by TMAO in strain MR1-R since the amount of *torF* transcript increased >60-fold when TMAO was added. In contrast, *torF* was poorly induced in strain SOR-3. These results clearly indicate that TorR is responsible for the strong induction of *torF* and confirm that TorR mediates TMAO signaling in *S. oneidensis*.



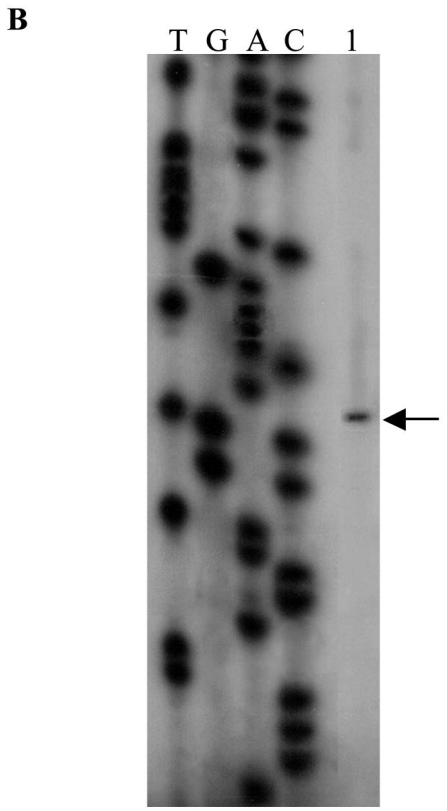


FIG. 4. (A) Nucleotide sequence of the *torF* promoter region. The transcription start site (+1) is indicated. The −10 region is indicated in bold. The ATG initiation codon and the direct repeats are underlined. Vertical bars above the sequence are positioned every 10 bases from the transcription start site. (B) Location of the transcription start point of gene *torF*. Labeled F5 primer, complementary to a *torF* internal sequence, was annealed to total RNA from *S. oneidensis* MR1-R cells grown anaerobically in the presence of TMAO and extended with RT (lane 1). The sequencing reactions were performed with the same primer as in the primer extension reaction. The samples were loaded on an 8% polyacrylamide–8 M urea electrophoresis gel. The arrow points out the transcription start site.

By using a plasmid-borne *torE'-lacZ* fusion, we have previously shown that the β-galactosidase activities increased almost 40-fold in the presence of TMAO in the wild-type context, whereas no significant increase was observed in the *torR* strain SOR-3 (6). Although these data are consistent with a strong TMAO induction of the *torECAD* operon mediated by TorR, they were indirectly assessed from a multicopy plasmid. To confirm *tor* operon activation and to compare it with that of *torF*, we carried out real-time PCR with *torC*-specific primers (C1-C2) and the cDNA samples generated for the *torF* expression study. As shown in Table 2, the *torC* induction factor was >20-fold in strain MR1-R, whereas it was close to 1 in strain SOR-3. The real-time PCR experiments thus confirm that the *torECAD* operon is activated by TorR, but the level of induction is somewhat lower for the *torECAD* operon than for the *torF* gene. This result was quite unexpected because the *torECAD* operon encodes the TMAO reductase respiratory complex and, thus, was supposed to be the main target of the TMAO response regulator TorR. In any case, the fact that *torF* is strongly induced by TorR suggests that the TorF protein plays a key role either in the TMAO respiratory system or in another TMAO-related pathway. A genome-wide transcriptional analysis has recently revealed that in *E. coli* the TorS/TorR phosphorelay system positively regulated the *tnaLAB* operon in addition to *torCAD*, but the TMAO induction factors, measured either from DNA arrays or from *lacZ* fusions, were clearly higher for *torCAD* than for *tnaLAB* (7).

TorF belongs to a new family of proteins of unknown function. The *torF* gene (*SO4694*) is a monocistronic unit encoding a putative protein of 245 residues with a calculated molecular mass of 26,998 Da. The amino acid sequence was compared with those of the proteins listed in the databases, and significant similarity was detected with several putative proteins encoded by various genomes of proteobacteria including *Azotobacter vinelandii* (Avin4116), *Bordetella pertussis* (BP1724), and *Caulobacter crescentus* (CC2658). However, no protein homologous to TorF is encoded by the related genome of *Vibrio cholerae* or by that of *E. coli*, and, in particular, no similarity was found with either TnaA or TnaB. Interestingly, one of the homologous proteins is encoded by a gene of *S. oneidensis* (*SO3502*), meaning that the *torF* gene might have been duplicated in this strain. So far, no biological function has been assigned to any of these homologues. These proteins could thus be classified in a new family of conserved proteins of unknown function.

Concluding remarks. The analysis of the DNA targets of the TMAO regulator TorR of *S. oneidensis* revealed that TorR recognizes highly specific operator sites containing a direct repeat of the sequence TTCATA. The TorR binding sites were only found in the promoters of *torECAD*, *torF*, and *torR*, and they allow TMAO induction of the *torECAD* and *torF* units and TMAO repression of the *torR* gene. Since the *torF* gene which encodes a protein of unknown function is coregulated with the *torECAD* operon encoding the TMAO respiratory system, we propose that TorF plays a specific role related to TMAO respiration. Future investigation will aim to define the function of TorF and of the other members of the TorF family.

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